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Published in:
Journal of Plant Biology Research

Publication date:
2015

Document version
Final published version

Citation for pulished version (APA):
Kjær, A., Yin, H., Verstappen, F., Bouwmeester, H., Ivarsen, E., Fretté, X., ... Jensen, M. (2015). Week long effects of applied stress on the production of artemisinin and precursors in *Artemisia annua* L. plants. Journal of Plant Biology Research, 4(2), 83-93.

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REGULAR ARTICLE

Week long effects of applied stress on the production of artemisinin and precursors in *Artemisia annua* L. plants

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ABSTRACT

The purpose was to examine the week long stress effect on full grown plants of *Artemisia annua* (*A. annua*) with attention to the content of artemisinin (AN) and its immediate precursors in young leaves. In two separate experiments, clonally propagated plants were stress treated either once or weekly for five weeks by sandblasting or spraying with salicylic acid, chitosan oligosaccharide, H₂O₂ or NaCl. Contents of AN and AN related compounds (AN-c) were analysed in leaf samples from the upper part of plants using triple-quadrupole UPLC-MRM-MS. Results showed that concentrations of the quantified AN and AN-c were statistically unchanged in response to multiple stress treatments, whereas plants responded to singularly applied stress treatments by a response wave of temporarily lowering the concentrations of several compounds related to the dihydroartemisinic (DHAA) branch of the biosynthetic pathway of AN. Response waves were similar among treatments, and only the timings varied. Results demonstrated that the applied stress initiated transient response waves through the DHAA related branch of biosynthetic pathway of AN. The speed of responses varied, with treatment of salicylic acid and chitosan oligosaccharide producing the fastest responses, followed by H₂O₂, NaCl and sandblasting.

Keywords: Artemisinin precursors, Short time effect, Stress, Triple-quadrupole UPLC-MRM-MS.

INTRODUCTION

The economically important sesquiterpene lactone artemisinin (AN) has only been found naturally occurring in *Artemisia annua* (*A. annua*) [1]. The glandular trichomes on the leaves are the primary site of production and storage of this anti-malarial compound and other secondary metabolites with several pharmaceutical and industrial uses [2]. Attempts to artificially synthesize AN or produce AN in engineered yeast and other bioengineered

target organisms have until recently proven difficult and not economically viable [3-6]. So in recent years, a considerable interest has been focussed on understanding the natural biosynthetic pathways of AN, the enzymes involved, and the underlying genetic expressions in order to find ways to increase the content of AN in plants or to produce it by biotechnology-based methods.

If “stress” is defined as conditions, which are suboptimal for the plants, the long term stress effect on content of AN and other AN related

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compounds (AN-c) has been demonstrated with numerous stress and stress simulating treatments, e.g. [7-16]. The results showed that it was possible to affect the concentrations of AN and some of the precursors by stress, often in a positive way, but, to our knowledge, no experiments have till now focussed on the short term effect of stress on the detailed composition of AN and its precursors. Stress is claimed to boost the expression of genes related to the biosynthetic pathway of AN, and thereby enhance the overall production of AN and AN-c, and further enhance the conversion of early stage AN precursors to later stage AN precursors and the end molecule AN itself.

The understanding of the biosynthesis of AN is under continuous elucidation, and the majority of the enzymatic and non-enzymatic steps in the pathway are now documented [8, 17-25]. The proposed biosynthetic pathway (Fig. 1) is based on the work of several researchers [3, 20, 26-32], and appears to be generally accepted. The most controversial part of the pathway is the possible pathway from artemisinic acid (AA), via arteannuin B and artemisitene, to AN (Fig. 1 (13)). Brown and Sy [33] performed a radiolabeling experiment and concluded that no direct path was found from AA to AN. But it was not stated which chemotype of *A. annua* was investigated, and results are as such not conclusive for all types of *A. annua*. The two different chemotypes of *A. annua* were proposed by Wallaart et al. [26] and comprised Type I, containing relatively high levels of AN and DHAA and low levels of AA, and Type II, containing relatively high levels of AA and low levels of AN and DHAA. Maes et al. [32] and Wu et al. [34] later demonstrated that a range of phytohormones initiated two different chemotypic expression patterns of key genes involved in the biosynthetic pathway of AN.

The current understanding of the possible stress induced intra- and interplant communication by signal molecules in *A. annua* is currently limited. To our knowledge, only Rapparini et al. [35] have investigated the possible interplant communication by emission of volatile organic compounds (VOC) in *A. annua*. However, in other species of *Artemisia* (*A. tridentate*, *A. cana*, and *A. douglasiana*) experiments have elucidated that the emissions of VOC play key roles in the defence response to wounding and the subsequent

damage induced by herbivores, often to the extent that the emission of VOC play a larger role than the internal signal transport within the individual plants [36-38]. Species of *Artemisia* are reportedly very active emitters of a broad spectrum of VOC, including methyl jasmonates, terpenoids and a range of green leaf VOC [37], but the exact mechanisms in interplant communication remain uncertain, though methyl jasmonate seem to play a key role [39-41].

There is a very limited knowledge on how the early changes in gene expressions induced by stress affect the detailed AN-c concentrations. Neither is it known if these early stress responses can be sustained over time if stress treatments are frequently applied or if the plant signalling mechanisms potentially get saturated as a consequence of adaptation in the plant to a new 'high stress normal'. The purpose of the present study was to investigate the early effects of selected applied stress treatments to large greenhouse grown *A. annua* plants, and study the different responses of plants treated once or multiple times. The analysed responses included highly detailed investigations of the changes in concentrations of AN and AN-c. The stress treatments were selected to represent a variety of stress types which was as broad as possible in order to create a diversity of responses.

MATERIALS & METHODS

Plant material: From a field population of seed propagated *Artemisia annua* (cv. 'Artemis', F2 seeds, Mediplant, Switzerland), two plants were selected as mother plant for clonal propagation by cuttings in a greenhouse (clones 1 and 2).

Tip cuttings were ca. 10 cm long and consisted of 4–5 internodes longer than 1 cm. Cuttings were rooted after 2–3 weeks, and potted in 3.5 L containers with Pindstrup No 2 peat moss (Pindstrup Mosebrug A/S, Denmark).

When plants were 9 weeks from the time of propagation and 80–110 cm in height they were transferred from the nursery to the experimental greenhouse, and plants used in experiment 1 (Ex1) were allowed to acclimatize for 7 days before the onset of the treatments, whereas plants used in experiment 2 (Ex2) were allowed 28 days before the treatments. Plants were 150–190 cm in heights at samplings. During the experiments, plants were

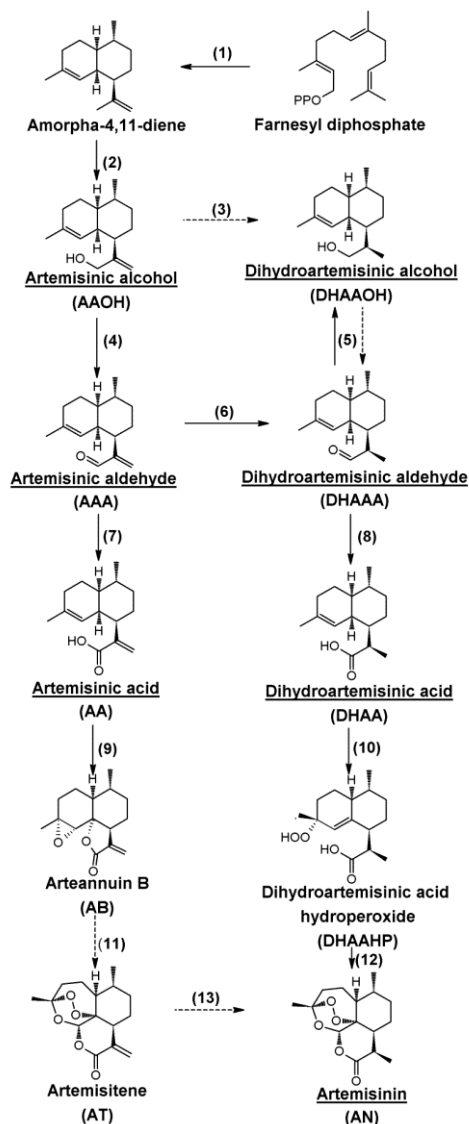


Figure 1. Biosynthetic pathway of AN. Names below the compounds with abbreviations used in text given in brackets. Compounds analysed in the present study are underlined. Conversions between molecules are marked with arrows. Commonly accepted conversions between molecules are marked with full arrows, whereas controversial conversions are marked with dotted arrows.

drip irrigated twice diurnally with a liquid fertilizer adapted for potted plants. Night temperatures ranged from 8 to 12 °C and day temperatures from 10 to 32 °C.

Experiments: The two experiments were carried out during April-May, 2010 in randomized complete block experimental designs with subsampling, and set up in a greenhouse divided into four separate self-contained compartments. In each compartment, two beds were established with 3 × 25 plants from each of the two clones.

Plants were 50 × 50 cm apart and individual beds were separated by 100 cm of gravel. Every second row of three plants were left as guard plants, allowing for 12 treatable subplots of three plants in each bed. In two compartments, stress treatments were performed five times at weekly intervals for five weeks (Ex1), and in the remaining two compartments, treatments were performed once (Ex2) 21 days after onset of treatments in Ex1. Treatments included spraying with NaCl (Salina) in 10 g L⁻¹ aqueous solution

(~171.12 mM), salicylic acid (S7401, Sigma Aldrich) in 1 g L⁻¹ aqueous solutions (~7.24 mM), hydrogen peroxide (Matas, 10%) at 1.0 % (~293.71 mM), chitosan oligosaccharide (Dalian Glycobio Ltd., China) in 1.0 g L⁻¹ aqueous solutions (molecular weight of mixed product was unknown), and sandblasting (Badger, Model 260-3, aluminium oxide particles). A manual garden vaporizer (Gardena) delivered 1 ml liquid per spray, and at the beginning of Ex1, 24 sprays covered the majority of a plant in a water film. To compensate for the growth during the five week treatment period, two additional sprays were added each week resulting in 32 sprays at last treatment of Ex1 and the single treatment of Ex2. Similarly, sandblasting was carried out for 2 × 15 seconds at the onset of the experiment (Ex1) and 2 × 20 seconds at the last treatments (Ex1 and Ex2). Treatments of both experiments were carried out during the late afternoon to minimize any sun scalding effect from water droplets on the leaf surface. To be able to compare results among different plants, an upper leaf was defined on each plant as the first leaf below the apex on the main stem with internodes longer than 2 cm. Leaf samples for analyses of AN and AN-c consisted of 4 entire main stem leaves collected around the upper leaf (two above and two below), frozen at -20°C, freeze dried for 48 hours, and stored at -20°C until extractions. Sampling of leaves in Ex1 was performed at 168 h after treatments, and sampling of leaves in Ex2 was performed just before treatments (0 h) and at 24 h, 48 h and 168 h after treatments. The 168 h sampling of Ex 1 was conducted 7 days after the 168 h sampling of Ex2. In Ex1, six plants from each treatment regime were sampled from each clone at each sampling time, and in Ex2 four plants were similarly sampled.

Sample preparation and UPLC-MRM-MS detection: Dry plant material was homogenized in a mixer mill (MM200; stainless steel ball Ø=7 mm; Retsch, Haan, Germany) and about 50 mg accurately weighed into an Eppendorf vial with 1.0 ml MeOH (HPLC grade; Th. Geyer, Germany), which was then briefly vortexed. The tubes were sonicated for 5 min. and centrifuged at 14000 rpm for 5 min. The supernatant was collected and filtered through a 0.45 µm syringe

filter into a brown HPLC glass vial. The pellet was re-dissolved in 0.5 mL MeOH and the procedure of sonication and centrifuging was repeated. The supernatant was again filtered into the vial, and stored at -20°C. Before measurements the samples were diluted 100 times with 20% acetonitrile in water (Biosolve, Valkenswaard, The Netherlands) containing 0.1% formic acid (Sigma-Aldrich, The Netherlands).

Targeted analysis of artemisinin and pathway intermediates was performed with a Waters Xevo tandem quadrupole mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was obtained on an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm; Waters) by applying a water/acetonitrile gradient to the column, starting from 5% (v/v) acetonitrile in water for 1.25 min and rising to 50% (v/v) acetonitrile in water in 2.35 min, followed by an increase to 90% (v/v) acetonitrile in water in 3.65 min, which was maintained for 0.75 min before returning to 5% acetonitrile in water using a 0.15 min gradient. Finally, the column was equilibrated prior to the next injection for 1.85 min using 5% acetonitrile in water. Operation temperature and flow rate of the column were 50°C and 0.5 mL min⁻¹, respectively. Injection volume was 5 µL. The mass spectrometer was operated in positive electrospray ionization mode. Cone and desolvation gas flows were set to 50 and 1000 L h⁻¹, respectively. The capillary voltage was set at 3.0 kV, source temperature at 150°C, and desolvation temperature at 650°C. The cone voltage was optimized for the different compounds using the Waters IntelliStart MS Console. Argon was used for fragmentation by collision-induced dissociation in the ScanWave collision cell. Quantification of compounds was done by multiple reaction monitoring (MRM). The optimized settings for MRM measurements are listed in Table 1. Both artemisinin and DHAA were a gift from Dafra Pharma (Belgium). DHAA was used by Chiralix (Nijmegen, the Netherlands) to synthesize the other precursors that were checked by NMR and were more than 98% pure. The reference standards were used for external calibration curves.

Data analyses: Graphs were prepared in Sigmaplot (2000 for Windows, ver. 6.00). Statistical analyses were performed in R ver. 2.11.1 [42]. Effects of stress treatments on AN and AN-c were analysed in a linear mixed model approach (lmer, lme4 package). Probabilities of significant difference from untreated controls collected at the same time were given for results within Ex1 (n=6) or Ex2 (n=4). All probabilities were given as $P > |t|$ in a MCMC algorithm.

RESULTS & DISCUSSION

Short term effect of stress treatments on concentrations of AN and AN-c: Results demonstrated a relatively consistent type of response to all types of stress within each of the two experiments. In the multiple treated plants (Ex1), none of the treatments resulted in significant differences in individual AN or AN-c concentrations or total compound concentrations at 168 h, as compared to controls (Figs. 2 and 3). In the once treated plants (Ex2), a common pattern in the responses was found, but with different timings among the treatments, suggesting the passage of a wave of responses through the biosynthetic pathway of AN (Figs. 2 and 3). These suggested response waves produced by all treatments included temporarily significant decreases in DHAA, DHAAA, DHAAOH, and AAOH, coinciding with non-significant decreases in the total compound concentrations, and non-significant tendencies for a later increase in AN concentrations as compared to control plants. SA and COS caused the fastest initiation of the response wave, H_2O_2 caused an intermediary fast initiation, and NaCl and SB caused the slowest initiations (Figs. 2 and 3).

The proposed wave was focussed on the “dihydroartemisinic acid” branch of the biosynthetic pathway (right-hand side of the diagram in Fig. 1), whereas the “artemisinic acid” branch seemed largely unaffected (Figs. 2 and 3). The response wave consisted of a drop in the concentrations in question, after which the concentrations returned to values similar to control values (particularly clear for 24 h in COS and 48 h in H_2O_2 (Figs. 2 and 3)). It is interesting to note that all significant changes of the proposed wave showed lower concentrations compared to controls, whereas no compounds showed higher concentrations in response to stress treatments.

This, and the transient non-significantly lower total concentration of all the quantified compounds (Fig. 2D), indicates a transient build-up of dihydroartemisinic acid hydroperoxide (DHAAHP) (Fig. 1), which was isolated and identified by Wallaart et al. [18], and is reported to undergo slow spontaneous autooxidation to AN [27]. Lommen et al. [43] suggested that DHAAHP accumulated in considerable quantities during maturation of the glandular trichomes, but to our knowledge DHAAHP has seldom been quantified in *A. annua* plants. Only Wallaart et al. [18] reported a yield of 40 mg DHAAHP per 100 g dry leaves corresponding to 0.25%, which, relative to the reported 0.5-0.8% content of AN, is a quite substantial amount. In the present study, the turnover rate from DHAA over DHAAHP to AN is impossible to quantify directly, but it is noteworthy that in the two fastest response waves (SA and COS), the concentrations of total compounds were close to having returned to the control values at 168 h, and the concentrations of AN were increased, though non-significantly. This indicates a relatively fast turnover from DHAA via DHAAHP to AN, though more studies are needed to verify this. In the early steps of the pathway (Fig. 1), the results suggested that relatively soon after the passage of the response wave, the concentrations of AN-c were replenished toward the levels of the control, though none of the affected compounds actually reached the control levels during the sampling period.

The experiment of multiple stress treatments (Ex1) only included one sampling for biochemical compounds at 168 h, and thus any possible wave pattern through time could not be discerned from the results (Figs. 2 and 3). However, it is noteworthy that none of treatments of Ex1 caused significant changes in any of the compounds, as compared to controls. If plants of Ex1 and Ex2 had responded with similar wave patterns, the late responding treatments (NaCl and SB) of Ex1 would have caused decreases in concentrations of DHAA, DHAAA, DHAAOH, and AAOH. As this was not observed, it is here suggested that single versus multiple stress treatments caused different response patterns.

The present report appears to be the first to propose the existence of a possible stress induced wave through this biosynthetic pathway of AN by

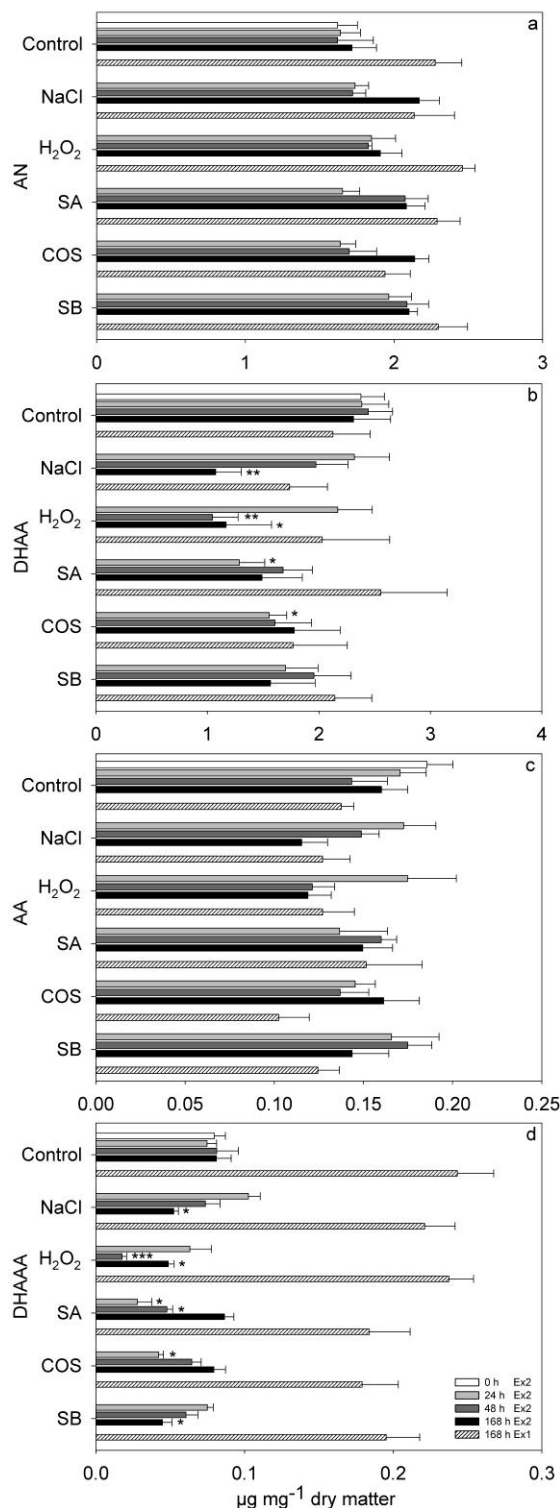


Figure 2. Concentration of compounds AN (a), DHAA (b), AA (c), and DHAAA (d) in control and stress treated plants. Treatments included NaCl, H₂O₂, salicylic acid (SA), chitosan oligosaccharide (COS), and sandblasting (SB). Obliquely shaded bars show results of Ex1 at 168 h. Results of Ex2 are shown as: White bars (0 h, only in control), grey bars (24 h), dark grey bars (48 h) and black bars (168 h). Results are shown as means ($n(\text{Ex1}) = 12$ and $n(\text{Ex2}) = 8$), and error bars indicate standard error of means. Asterisks indicate probability in a linear mixed model of difference from controls sampled at the same time within each experiment. All probabilities were given as $P > |t|$ in a MCMC algorithm (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

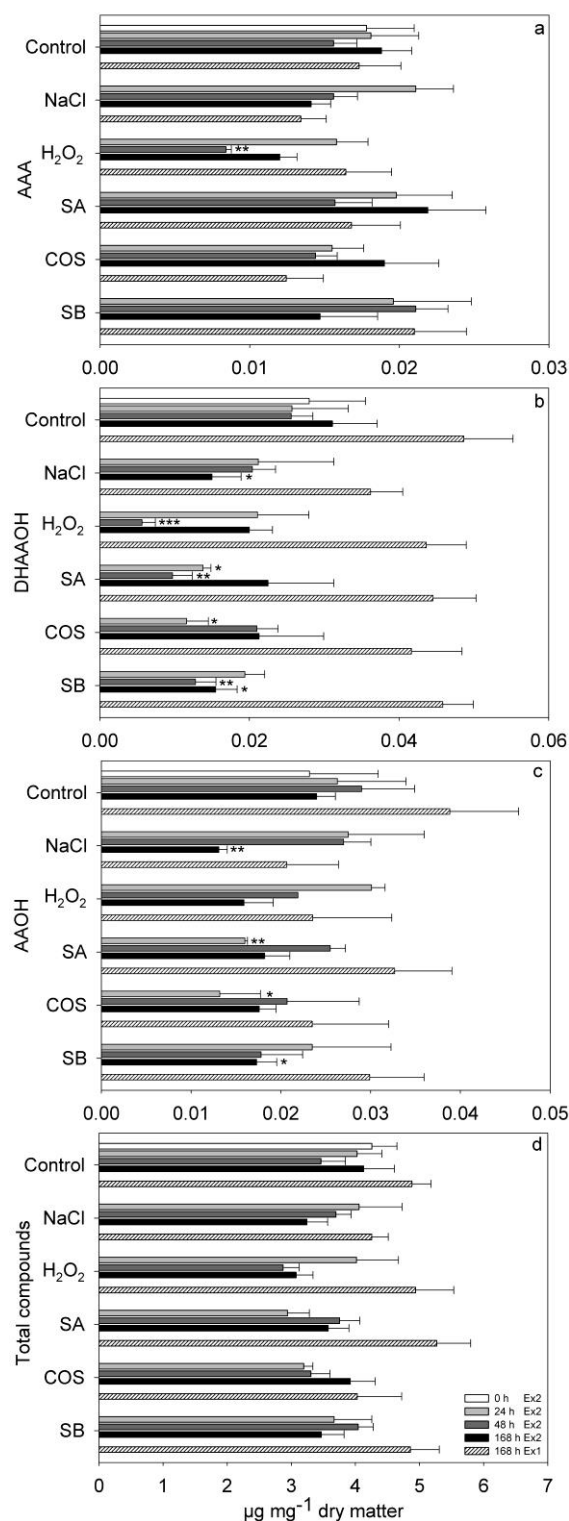


Figure 3. Concentration of AAA (A), DHAAOH (B), AAOH (C), and total compounds (AN + AN-c) (D) in control and stress treated plants. Treatments included NaCl, H_2O_2 , salicylic acid (SA), chitosan oligosaccharide (COS), and sandblasting (SB). Obliquely shaded bars show results of Ex1 at 168 h. Results of Ex2 are shown as: White bars (0 h, only in control), grey bars (24 h), dark grey bars (48 h) and black bars (168 h). Results are shown as means ($n(\text{Ex1}) = 12$ and $n(\text{Ex2}) = 8$), and error bars indicate standard error of means. Asterisks indicate probability in a linear mixed model of difference from controls sampled at the same time within each experiment. All probabilities were given as $P > |t|$ in a MCMC algorithm (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

a detailed analyses of AN and AN-c. However, some aspects of the study have parallels in previous studies. Lei et al. [44] sprayed COS on 45 days old clonally propagated plants, and collected leaves from an unreported position of the plants. They observed transient increases in concentrations of AN and DHAA, with optimum concentrations at 48 h and 24 h, respectively, whereas AA showed no significant changes. Liu et al. [11] wounded 40 cm tall plants, and collected the middle leaves, and observed small, insignificant rises in concentration of AN in the 4–48 hours after treatments, but these were undetectable again at 72 h. Banyai et al. [12] soil drenched 45 days old wild type plants in a GA₃ solution, and collected leaf samples comparable to the ones collected in the present experiments, and demonstrated a transient significant rise in AN at 24 h and 48 h, after which AN declined, but rose significantly again at 14-28 days. In the present study, several treatments (NaCl, SA, COS and SB) showed similar, but non-significant, trends of increasing AN concentrations at different sampling times (Fig. 2), though no signs were observed of these being of a transient nature as demonstrated by Lei et al. [44], Banyai et al. [12] and Liu et al. [11]. The COS induced increase in DHAA concentrations with optimum at 24 h observed by Lei et al. [44] were not confirmed in this study, which demonstrated a decrease at 48 h. This and the similar observations of the transient decreasing effect of most treatments on the concentrations of DHAA, DHAAA, DHAAOH, and DHAAOH have not previously been reported in literature.

Although the resolution of the time line was relatively coarse with only four sampling points (0 h, 24 h, 48 h, and 168 h), it was nevertheless possible to distinguish that some treatments initiated the wave sooner than others. The fastest responses were caused by SA and COS, indicating that these stress agents had a direct influence on the pathway. Both SA and COS have previously been demonstrated to elicit fast responses [7,44]. H₂O₂ showed an intermediary fast response, indicating that the applied H₂O₂ may have contributed to the internal ROS (Reactive Oxygen Species) communication and reactions in the plants. The two stress treatments, which in theory should act as external stress agents (NaCl and SB), produced the slowest responses, possibly

indicating the initiation of a more complex signal way, compared to the stress agents acting directly on the pathway.

Effect of single versus multiple stress treatments:

The experiment elucidating the short term effect of a single stress treatment (Ex2) was performed parallel to the experiment investigating the effect of multiple stress treatments (Ex1)(reported more extensively in Kjaer et al. [16]), and a comparison between the two experiments was performed. However, the experimental setup did not allow for a direct statistical evaluation of the comparison, as it did not include a complete randomisation, and furthermore the sampling times of the two experiments were separated in time by one week. Nevertheless some interesting trends were noted. Observing the waves of response in single treatment experiment (Ex2) prompted us to anticipate significant decreases in concentrations of DHAA pathway related compounds in the slow responding treatments by NaCl and SB in the 168 h samples from Ex1, but Ex1 results showed no significant decreases in concentration of any of the individual AN-c in response to treatments (Figs. 2 and 3). Taking all reservations into account, we thus hypothesise that a single stress treatment had a higher impact on the defence apparatus of the plants, than in multiple stressed plants, possibly because the multiple treatments led to an adaptation to the treatments. Adaptation to stress has, to our knowledge, not previously been demonstrated in *A. annua*.

Figures 2 and 3 show that when plants were treated multiple times (Ex1), all treatments, including controls, showed several-fold higher concentrations of DHAAOH and DHAAA (except SA), and markedly lower concentrations of AA (except NaCl) and AAA (except Control, H₂O₂ and SB) at 168 h, as compared to the singularly treated plants of Ex2 at 168 h. The interesting part of this observation was that the untreated control plants from the two experiments exhibited equally large differences as the treated plants. Going through the experimental setups and executions produced no obvious explanations to this enigma. The two experiments were conducted in a greenhouse divided by airproof glass walls into four compartments (blocks), and the results from the two blocks within each experiment were virtually indistinguishable from each other. The

only tangible difference among the experiments, apart from the number of treatments, was that the Ex1 plants were sampled one week later than Ex2, but as sampling of leaves for analyses was conducted at the same developmental stage of the shoot in both experiments, this cannot explain the observed difference. The fact that untreated control plants contained different concentrations of several AN-c, depending on which number of treatments the plants in their immediate vicinity received (Ex 1 and Ex2, respectively), lead us to question the presumption that only direct contact with the treatments changed the AN related profile of the plants. This facilitates the suggestion that *A. annua* might possess a mechanism of communication between individual plants by emission of volatiles, and thus prompting the control plants to react to the stress treated plants from the same block. This has not previously been demonstrated in *A. annua*, but has been demonstrated in other plant species, e.g. [45-46] and in other species of *Artemisia* [36-38, 41].

CONCLUSION

The present investigation for the first time demonstrate changes in the detailed composition of many of the precursors involved in the biosynthetic pathway of AN in response to a broad range of stress types. Results indicated that single stress treatments sent a transient response wave through the dihydroartemisinic acid branch of the pathway of AN. The response wave included simultaneous significant transient lowering of the concentrations of DHAA, DHAAA, DHAAOH, and AAOH, non-significant decreases in the total concentrations of compounds, and non-significant increases in concentrations of AN. The speed of responses varied, with treatment of SA and COS producing the fastest responses, followed by H₂O₂, NaCl and SB. A transient build-up of dihydroartemisinic acid hydroperoxide was suggested to be part of these changes, but not measured here. The implications of the present findings are primarily of scientific interest, and support the contemporary understanding that stress can indeed affect the concentrations of AN and AN-c. The understanding that different types of stress have differently timed response patterns may prove valuable in future research in the stress signalling pathways within *A. annua*. The suggestion that the plants may also have volatile

signalling pathways, should prompt future research in stress treatments of *A. annua* to design and execute stress experiments in ways to isolate the effect of the *applied* stress from the *perceived* stress of the individual plant.

ACNOWLEDEMENTS

The authors wish to thank field technicians Astrid Bergman, Birthe Flyger and Jens Barfod for assisting in maintaining and sampling the experimental plants. We also acknowledge the valuable input to the microscopy techniques by laboratory technician Annette S. Brandsholm. Dr. Willemien Lommen, Wageningen University, The Netherlands, and the members of the Artemisia Project Group, Denmark have been highly valued sparing partners. This work was supported by The Strategic Research Council, Denmark [FI. 2101-08-0048].

Abbreviations:

Artemisinin (AN)
AN related compounds (AN-c)
Dihydroartemisinic acid (DHAA)
Artemisinic acid (AA)
Dihydroartemisinic aldehyde (DHAAA)
Artemisinic aldehyde (AAA)
Dihydroartemisinic alcohol (DHAAOH)
Artemisinic alcohol (AAOH)
Dihydroartemisinic acid hydroperoxide (DHAAHP)
Artemisia annua (*A. annua*)
Salicylic acid (SA)
Chitosan oligosaccharide (COS)
Sandblasting (SB)

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